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Authors

Martins-Green, M
Bissell, MJ

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Localization of 9E3/CEF-4 in Avian Tissues: Expression Is Absent in Rous Sarcoma Virus-induced Tumors But Is Stimulated by Injury

Manuela Martins-Green and Mina J. Bissell

Cell and Molecular Biology Division, Lawrence Berkeley Laboratory, Berkeley, California 94720

Abstract. The avian gene 9E3/CEF-4, a member of the superfamily of genes that includes *KC* and *gro*, is expressed abundantly in exponentially growing cultures of chick embryo fibroblasts (CEFs) and at high levels in CEFs transformed with Rous sarcoma virus (RSV). The product of this gene is a secreted protein that has homologies and structural similarities to inflammatory mediators. The function of 9E3 is obscure and its expression *in vivo* has not yet been investigated. We studied by *in situ* hybridization and RNA blots the pattern of 9E3 mRNA distribution in the wings of normal, wounded, and RSV-infected newly hatched chicks. We found that the message for 9E3 is high in specific tissues in normal wings; whereas connective tissue, tendon, and bone express the gene, muscle fibers, endothelium, epidermis, and bone marrow do not. The distribution coincides with that of interstitial collagen. Wounding results in marked elevation of the mRNA within the granulation tissue formed during healing and in adjacent tissues, especially those showing neovascularization. Similar elevation of mRNA occurs immediately adjacent to

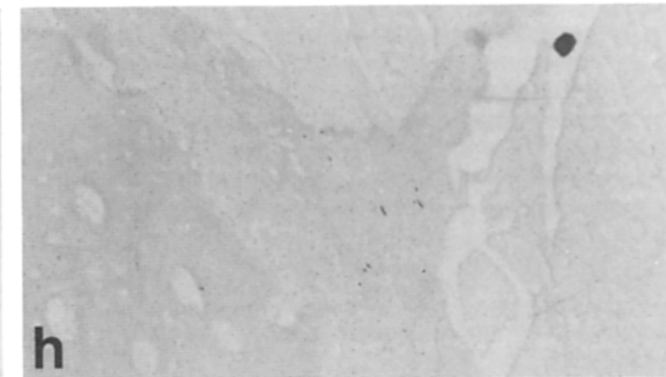
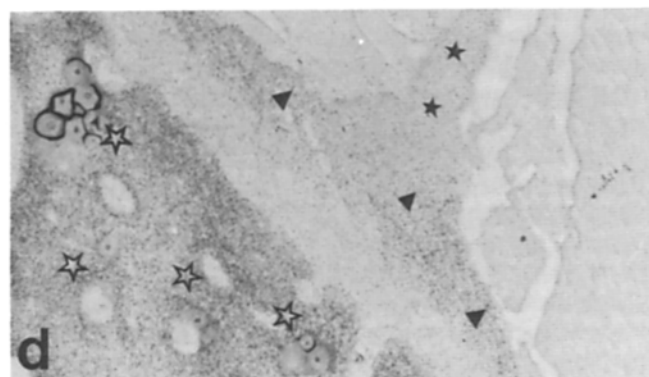
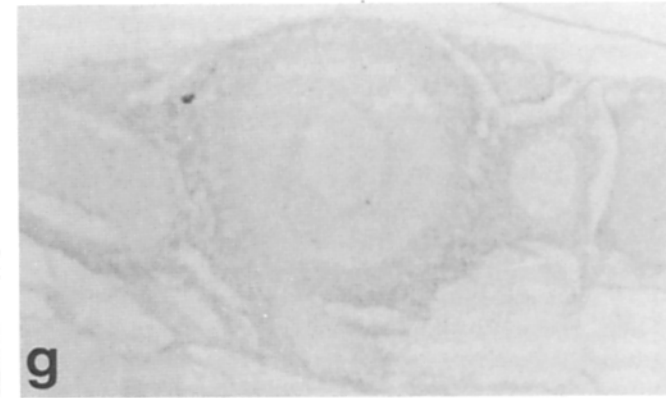
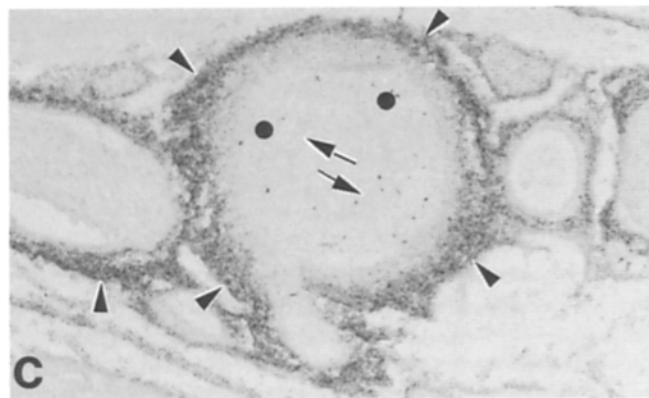
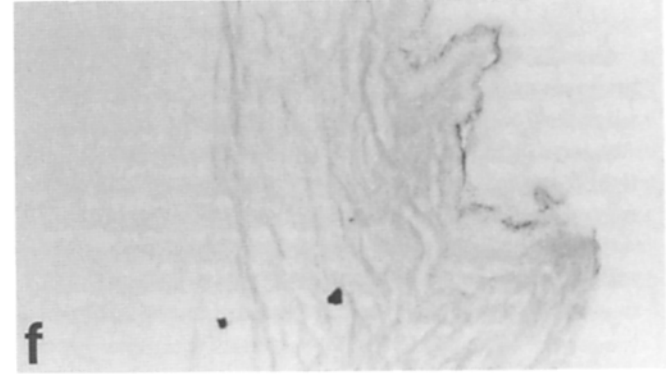
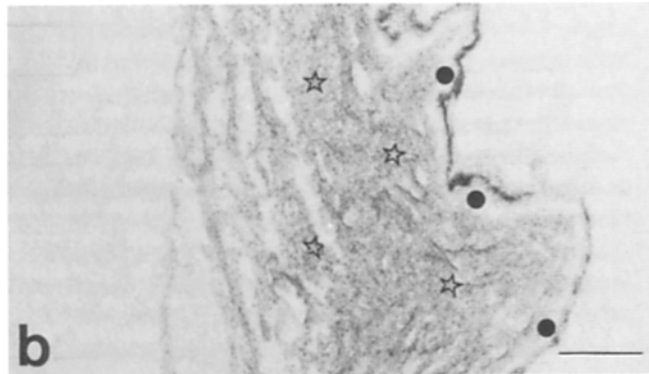
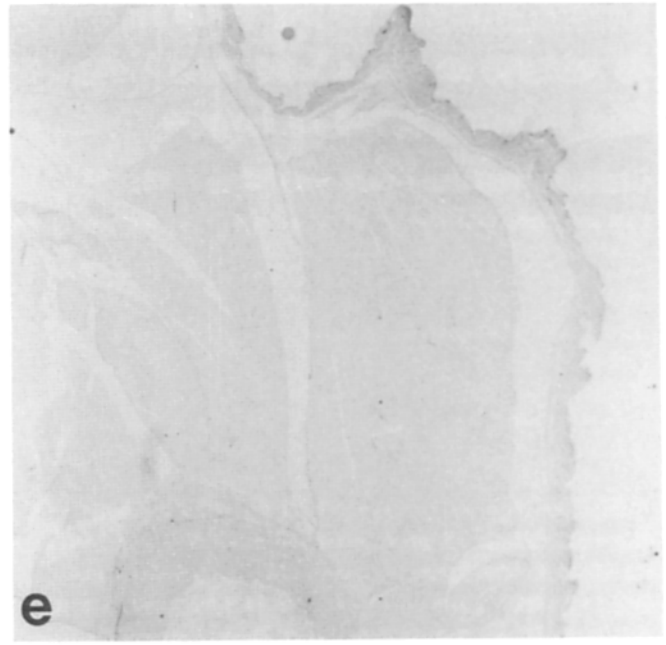
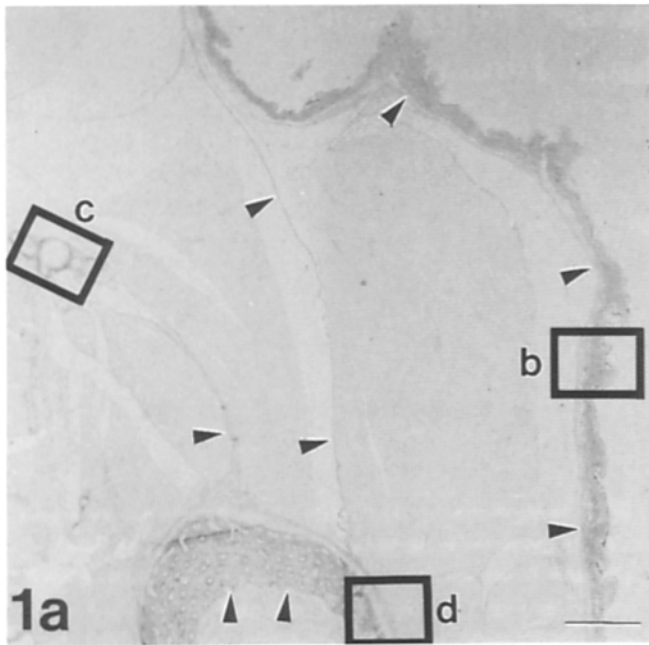
RSV tumors but, surprisingly, the tumor tissue itself shows no detectable levels of this message. Cells explanted from the tumors and grown in culture also show no expression of 9E3, in marked contrast to the very high level found in similarly cultured RSV-transformed CEFs. These results show that there are intrinsic differences between transformed embryonic cells in tissue culture and RSV target cells in the hatched chick. However, the expression of the gene in the periphery of tumors leaves open the possibility that 9E3 may still be involved in RSV carcinogenesis. The abundant expression of 9E3 in normal tissues indicates that the product of this gene plays a normal physiological role in tissues growing by cell division, perhaps as a growth regulator. The elevated expression of 9E3 in areas of neovascularization, makes it possible that the product of this gene could act as an angiogenic factor. Finally, expression in conjunction with high collagen levels and in wounded tissues may point to a role in wound response and/or repair, possibly via alteration of extracellular matrix.

A number of growth-related genes have been isolated recently whose cDNAs show extensive sequence similarities. Several of these have been cloned independently and have been given a variety of names. The first of these genes to be isolated, *KC* (Cochran et al., 1983), is induced by PDGF in BALB/C-3T3 cells and may play a role as a mediator of the mitogenic response to PDGF. Another gene, whose product is melanoma growth-stimulating activity (MGSA),¹ was isolated and cloned from human melanoma cells by Richmond and colleagues (Richmond et al., 1983, 1985, 1988; Richmond and Thomas, 1988); it acts as a growth stimulator for melanoma cells. More recently, two other cDNAs have been cloned, one of which, the hamster *gro* gene, was isolated from a Chinese hamster fibroblast cell

line, CHEF/16, that is highly tumorigenic. This gene is tightly regulated by the growth status of non-transformed CHEFs but is overexpressed in the CHEF/16 line (Anisowicz et al., 1987). The second of these genes, human *gro*, was isolated from human tumor cells and shows a pattern of expression similar to that of the hamster gene. Human *gro* has a nucleotide sequence identical to that of the gene for MGSA (Anisowicz et al., 1987) and the *KC* gene (Oquendo et al., 1989). The latter investigators generated a phylogenetic tree for this group of genes that places platelet factor 4 (PF4) near the root.

In addition to the nucleotide sequence homology shared by these genes, their products are secretory proteins that have homologies and structural similarities to inflammatory mediators. These include: (a) β -thromboglobulin (Begg et al., 1978), (b) γ -IP 10 (Luster et al., 1985); (c) connective tissue-activating peptide III (CTAP-III; Castor et al., 1983; Anisowicz et al., 1987), (d) a monocyte-derived factor (now

1. *Abbreviations used in this paper:* MGSA, melanoma growth-stimulating activity; RT, room temperature; T-CEF, RSV-transformed chick embryo fibroblasts; TGF, transforming growth factor.



called IL-8) that is chemotactic for neutrophils (Yoshimura et al., 1987) and secreted by activated leukocytes (Schmidt and Weissmann, 1987); and (e) MIP-2 (Wolpe et al., 1989). Therefore, this new family of genes and factors may be important in cell proliferation and transformation, and also in inflammation and/or wound healing.

The avian gene, 9E3/CEF-4, belongs to this family; it was isolated from cultured RSV-transformed chick embryo fibroblasts (T-CEFs) by Sugano et al. (1987) and Bedard et al. (1987). This gene (henceforth referred to as 9E3) is transcriptionally regulated and encodes for a 9.0-kD secretory protein with homologies to the inflammatory mediators platelet basic protein, platelet factor-4, and γ -IP-10. In serum-starved cultures of confluent chick embryo fibroblasts (CEFs), 9E3 is expressed at very low levels; when stimulated by addition of serum factors, its expression is enhanced. The gene also is abundantly expressed in cells plated at low density but declines as the cultures become confluent. These two observations indicate that the expression of 9E3 is low when cells are cultured under conditions that do not favor growth. When CEFs are transformed with RSV in culture, 9E3 mRNA is greatly enhanced and the high expression is maintained. Indeed, it was this overexpression in transformed CEFs that led to isolation of the gene (Sugano et al., 1987; Bedard et al., 1987).

We have been investigating the relationship between wounding and RSV tumorigenicity. We showed previously that newly hatched chicks, when injected with RSV, develop tumors only at the site of injection (primary tumor) or when a wound is inflicted elsewhere (wound tumor), in spite of the fact that virus is circulating in the blood (Dolberg et al., 1985; Sieweke et al., 1989). This observation suggests that factors in addition to pp60^{v-src} may be involved in RSV tumorigenicity. The association of 9E3 with RSV transformation in culture, and its homology to inflammatory mediators, suggested that the product of this gene might be one such factor. Therefore, we investigated the expression of this gene in the wings of newly hatched chicks after wounding and after infection with RSV. Our results indicate that 9E3 plays a physiological role in normal tissue development and may be important for wound response and/or repair. However, 9E3 mRNA is not present in tumor cells themselves, suggesting that this gene is not involved in the maintenance of RSV-induced tumors. Nevertheless, the gene is expressed abundantly at the growing boundary of the tumors, indicating that it could be important in creating a permissive environment for tumor growth.

Materials and Methods

Cell Cultures

Fibroblasts were isolated from 10-d-old chick embryos as described previ-

ously (Bissell et al., 1977). Briefly, cells were first seeded in 100-mm plates in 2% calf serum and 1% chicken serum. They were grown for 4–5 d and then seeded in uncoated 35-mm tissue-culture dishes and cultured in medium containing 4% calf serum and 1% chicken serum for varying periods of time. Transformed fibroblasts were obtained by infecting primary cells with virus and subculturing them once in the same medium and under the same conditions as above. Tumor cells were explanted from 14-d primary tumors (by mincing pieces of tumor tissue with two sterile razor blades), plated directly onto culture dishes and grown, for varying periods of time, as described above.

Preparation of Tissues for In Situ Hybridization and Histological Stainings

Newly hatched chicks (5 d old) were injected intramuscularly in the middle portion of the left wing with 1×10^3 focus-forming units (f.f.u.) of RSV (Schmidt-Rupin or Prague C strains) in 100 μ l of medium 199. 5 d later, a metal clip or a suture was inserted between the ulna and radius of the right wing (Dolberg et al., 1985; Sieweke et al., 1989). The wings containing primary tumors were removed 11–12 d after injection and wings with wound tumors or wound reactions were removed 11–12 d after injury. The feathers and clip were removed from each wing and the mid-portion of the wing was placed in 4% paraformaldehyde in PBS, pH = 7.4 for 4 hours at room temperature (RT). The tissue was then placed in copious amounts of decalcifying solution (5% formic acid, 2.5% formaldehyde in PBS, pH = 7.4) for 24 h in a rocker. At this stage, the wing pieces were cut in half and placed back into decalcifying solution for an additional 48 h, with one change of solution. The tissues were then incubated in 0.1% glycine in PBS for 1 h at RT, dehydrated in ethanol, embedded in paraffin, and sectioned (4- μ m-thick sections). Adjacent sections were used for in situ hybridization and for hematoxylin/eosin and Masson's Trichrome stainings (Luna, 1968).

Immunolabeling

von Willebrand (vW) Factor-like Molecule. Sections were deparaffinized twice in xylene for 10 min each, hydrated in a descending ethanol series, rinsed in double-distilled water, and incubated in 0.1% trypsin in 0.1% CaCl₂ for 50 min at 37°C. The sections were washed in TBS + 0.1% BSA, incubated with TBS + 1% BSA for 10 min, and then with the antibody against vW factor (made in rabbit against human vW factor, Dako Corp., Santa Barbara, CA) diluted 1:100 in TBS + 1% BSA overnight at 4°C. The primary antibody was washed with TBS + 0.1% BSA three times for 10 min each at RT and then incubated with a goat anti-rabbit FITC secondary antibody (Zymed Laboratories, San Francisco, CA; 1:25 in TBS + 1% BSA) for 1 h at RT. This Ab was washed three times for 10 min each with TBS + 0.1% BSA and mounted in buffered glycerol containing *p*-phenylene-diamine (Johnson and Nogueira Araujo, 1981).

p19^{gag}. Tumor cells in culture were labeled with an antibody to p19^{gag} (a group-specific antigen protein of the virus) using the procedure described previously by Stoker and Bissell (1987).

Isolation of RNA

Preparation of Slot and Northern Blots. RNA was extracted from normal and transformed CEFs in culture using 0.05 M sodium acetate buffer, pH 5.2, containing 0.05 M EDTA and 0.5% SDS (extraction buffer). Fibroblasts in 100-mm culture dishes were washed once with PBS and covered with 2 ml of extraction buffer for 5 min. The lysates were removed into a falcon tube and immediately mixed with equal volumes of phenol (equilibrated to pH = 5.2 with acetate buffer) followed by phenol chloroform (1:1) and finally chloroform alone. The RNA was precipitated overnight at -20°C with 0.1% 3 M Na acetate and 2.5 vol of cold 200-proof ethanol; after centrifugation, the pellet was resuspended in sterile water. RNA was isolated

Figure 1. Cross-sections through the wing of a 2-wk-old chick. In situ hybridization was performed using a probe for 9E3 mRNA (a–d) and for the pGEM1 vector (e–h). (a) Low magnification of wing tissues; dark areas (arrowheads) represent sites of hybridization of the probe with 9E3 mRNA. (b) Detail of box b in (a) showing hybridization in the dermis of the skin (asterisks), but absence in the epidermis (dots). (c) Detail of box c in (a) showing 9E3 expression in the connective tissue around blood and lymph vessels (arrowheads) but not in the smooth muscle (dots) and endothelial cells (arrows). (d) Detail of box d in (a) showing hybridization in the bone (asterisks), periosteum (triangles) and tendon (stars). (e) Section consecutive to that shown in (a) but hybridized with a control probe (pGEM1 vector). Notice that the skin, bone, tendon, and connective tissue of blood and lymph vessels do not appear dark, indicating absence of hybridization. (f–h) Enlargements of areas identical to those boxed in a for comparison with b through d showing in detail that the control is negative. Scale bar, 500 μ m for a and e and 50 μ m for b–d and f–h.

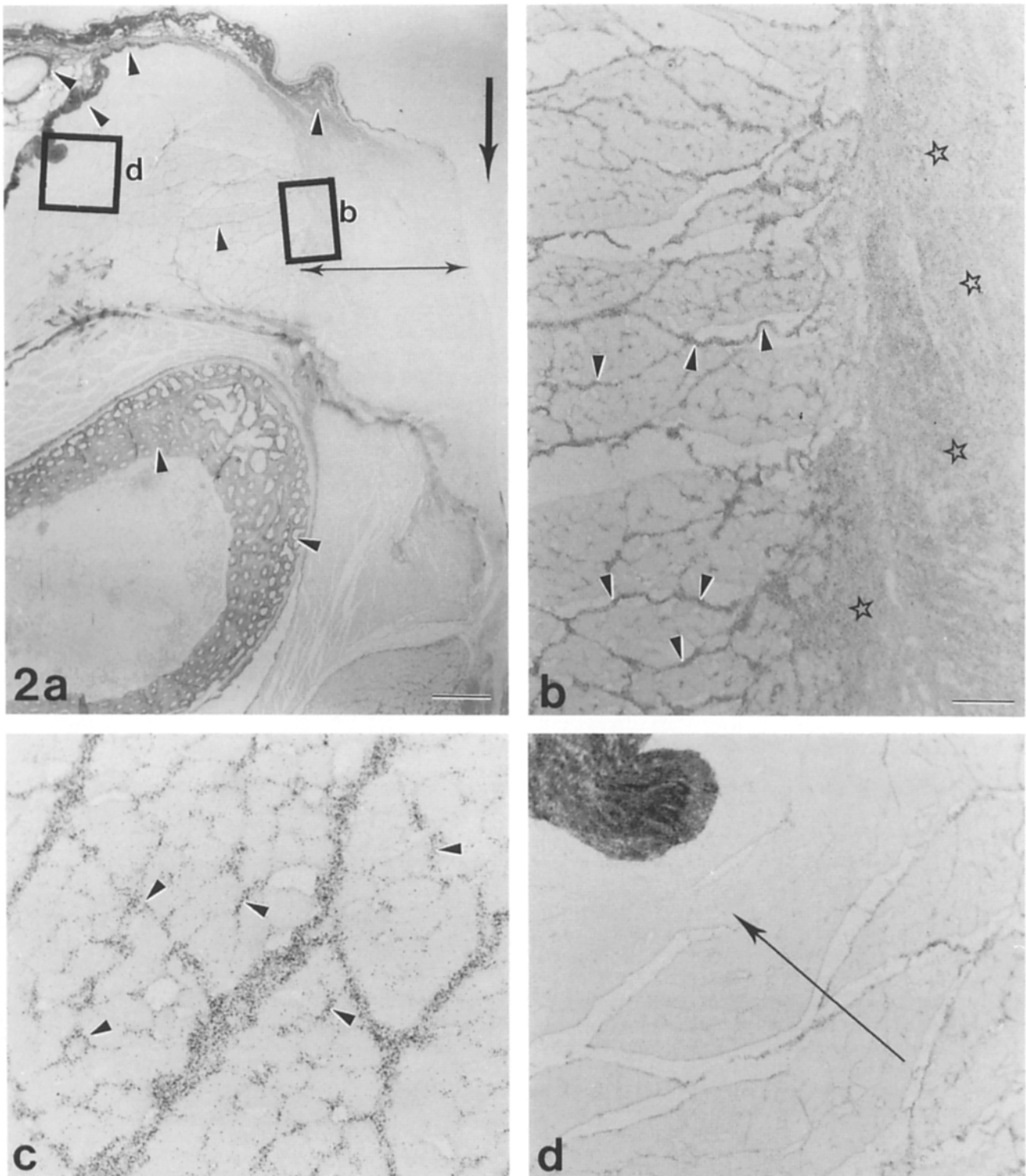


Figure 2. Cross-section through the wing of a 2-wk-old chick after wounding by insertion of a clip 11 d before. In situ hybridization was performed using a probe for 9E3 mRNA. (a) Low magnification showing the site where the clip was inserted (*thick arrow*), as well as granulation wound tissue (*double-headed arrow*) and adjacent tissues. Dark areas (*arrowheads*) represent sites of hybridization of the probe with the 9E3 mRNA. (b) Enlargement of box "b" in (a) showing heavy hybridization in the granulation tissue (*asterisks*) and in the connective tissue around the muscle blocks (*arrowheads*). (c) Higher magnification to better illustrate the increase in 9E3 mRNA around muscle blocks and individual muscle fibers (*arrowheads*; compare with Fig. 3 c). (d) Detail of box "d" in (a) illustrating that 9E3 mRNA decreases with distance away from the wound site (*arrow* points away from wound). Scale bar, 500 μ m for a and 50 μ m for b–d.

from tissues by freezing and grinding the tissue to a powder in liquid N₂, mixing with the extraction buffer and following the procedure described above. The formaldehyde agarose gel electrophoresis and transfer to Hybond-N (Amersham Corp., Arlington Heights, IL) were done according to Maniatis et al. (1982). After transfer, the filter was baked in a vacuum oven at 80°C for 2 h. For the slot blot, 6 µg of RNA was denatured in 200 µl of 6× SSC containing 20% formamide at 65°C for 15 min, cooled on ice for 15 min and applied onto the Hybond-N filter prewetted with 6× SSC using a Manifold II apparatus (Schleicher & Schuell, Keene, NH).

Preparation of the DNA Probe and Hybridization of Blots. The cDNA clone for 9E3 (kindly provided by H. Hanafusa, Rockefeller University, NY) was expanded in *Escherichia coli* HB 101 extracted with alkaline lysis buffer, isolated by CsCl gradient centrifugation, extracted with phenol, phenol:chloroform, and chloroform, and then ethanol precipitated. The DNA probe was prepared with ³²P dCTP by random primer extension (Feinberg and Vogelstein, 1984) overnight at RT and separated from the free nucleotides in a 1-ml G-50 Sephadex spin column (Pharmacia Fine Chemicals, Piscataway, NJ). The Hybond-N filters were placed in a plastic bag, 10 ml prehybridization solution (45% formamide, 5× SSC, 1× Denhardt's solution, 20 mM Na₂P₂O₄, pH 7.0, 10% dextran sulfate, 100 µg/ml calf thymus DNA heated to separate the strands, 0.1% SDS) was added and the bag was sealed and incubated for 5 h at 42°C. At the end of this period 1× 10⁶ cpm of probe/ml of hybridization solution was added, the bag resealed and incubated at 42°C for 18 h. The filters were washed in 2× SSC + 0.1% SDS for 1 h at RT in the shaker, changing solution once. The final wash was done in 0.1× SSC + 0.1% SDS at 68°C for 30 min. The filters were air dried, wrapped in Saran Wrap, and exposed to Kodak XAR-5 film with an intensifying screen at -70°C for 48 h.

Preparation of the RNA Probe and In Situ Hybridization. The cDNA clone for 9E3 was recloned into the Eco RI site in pGEM1 transcription vector (Amersham Corp.), such as to give antisense transcription from the T7 polymerase site. The plasmid was linearized with the Bam HI restriction enzyme, purified by extraction with phenol, phenol:chloroform, and chloroform, and precipitated with 0.3 M Na acetate and 2.5 vol of ethanol. We used the procedure developed by R. Schwarz (unpublished results) for the in vitro transcription and in situ hybridization. Briefly, for in vitro transcription, 1 µg of plasmid cDNA was transcribed at 37°C for 1 1/2 h in the presence of 100 µM [³H]ATP and [³H]UTP (Amersham Corp.) each, 1 mM GTP and CTP each, 120 mM DTT, and 100 U of T7 polymerase (Amersham Corp.). The probe was separated from the free nucleotides in a 6-ml Sephadex G-50 column equilibrated with 50 mM Tris pH 8.0, 15 mM NaCl, and 1 mM EDTA. For in situ hybridization of fibroblast cultures, the cells were fixed, hybridized, washed, allowed to dry, covered with 1:1 H₂O:NTB-2 liquid emulsion (Eastman Kodak Co., Rochester, NY) and dried for 2 h in the dark. Exposure was carried on at 4°C for 4 wk in tightly sealed boxes containing desiccant. The emulsion was developed in D-19:H₂O (1:1) for 5 min, rinsed, fixed in Kodak fixer for 5 min and washed in running water for 15 min. The cells were stained with Wright staining: H₂O (1:10) for 10–15 min, rinsed with H₂O, air dried, and observed.

4-µm-thick wing sections were cut and collected on organosialinized slides (Tourtellotte et al., 1987) and baked overnight at 40°C. They were deparaffinized twice in xylene 10 min each, hydrated in a descending ethanol series, rinsed in PBS for 5 min, fixed for 10 min with 4% paraformaldehyde in PBS (pH 7.4), incubated in 0.1 M glycine in PBS for 5 min, washed in PBS and dehydrated with ethanol. The sections were allowed to dry completely and then each two sections were covered with 100 µl hybridization solution (50% deionized formamide, 5 mM EDTA, 0.3 M NaCl, 20 mM Tris-HCl pH 8.0, 1× Denhardt's solution, 10% dextran sulfate) containing 1 × 10⁶ cpm of probe and 40 µg of tRNA. The sections were covered with coverslips (previously baked at 150°C for 2 h) and incubated in a sealed, humid chamber at 55°C for 14 h. The slides were dipped in PBS to remove the coverslips and washed with gentle shaking as follows: 2× SSC for 30 min at RT, 2× SSC + 0.1% Triton X-100 at RT, 0.1× SSC for 30 min at RT and 0.1× SSC + 0.1% Triton X-100 for 1 h at 55°C. The slides were allowed to dry completely before being covered with NTB-2 liquid emulsion. Exposure was done as described above.

Results

Expression of 9E3 in Normal and Wounded Wing Tissues

The expression of 9E3 in vivo has not been investigated previously. It thus was important to establish whether or not

normal tissues expressed this gene. We performed in situ hybridization on sections of wings of newly hatched chicks (Fig. 1) and found that the mRNA was present mainly in the dermis layer of the skin (Fig. 1 b) and in the connective tissue around blood vessels (Fig. 1 c), muscle blocks and individual muscle fibers (not shown, but see Fig. 2 c). In addition, 9E3 is present in tendon and bone, including the periosteum (Fig. 1 d), and in the feather germ layer (not shown). It is not expressed in epidermal cells (Fig. 1 b), endothelial cells (Fig. 1 c), bone marrow (not shown) or within muscle fibers (see Fig. 2 c). Controls, prepared by hybridizing consecutive sections with a probe transcribed from the vector, were negative (Fig. 1, e–h).

To investigate the role of 9E3 in wounding and to provide a control for study of wound tumor formation (see below), we examined wings of healthy birds that had been wounded by a metal clip (Dolberg et al., 1985) or by insertion of a suture thread (Sieweke et al., 1989). In situ hybridization was performed 11–12 d after wounding. This particular time was chosen to facilitate comparison with RSV-induced tumors that develop ~10 d after injection or wounding (see below). Expression of 9E3 was enhanced in the vicinity of wound infliction (Fig. 2); it was very high in the connective tissue around individual muscle fibers adjacent to the granulation tissue of the wound (Fig. 2, b and c) but it was at normal levels away from the wound site (Fig. 2 d). Within the granulation tissue itself, expression also was elevated in the area close to the interface with the muscle where neovascularization was intense (Fig. 2, b and c, see also Figs. 4 d and 6, d–f). Controls (not shown) were negative.

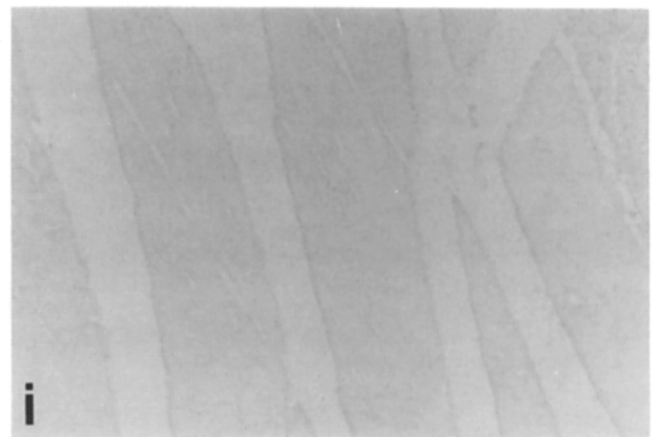
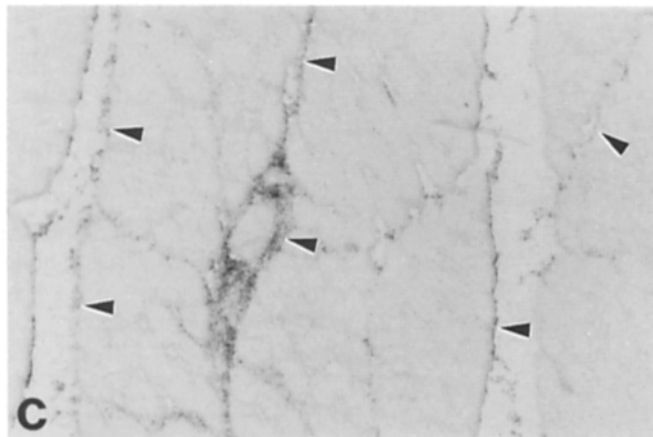
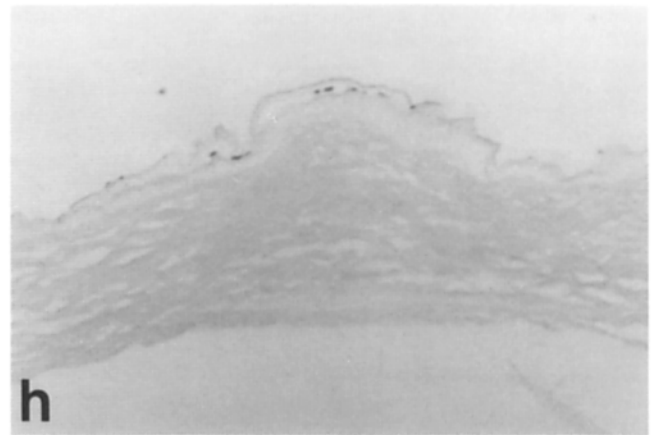
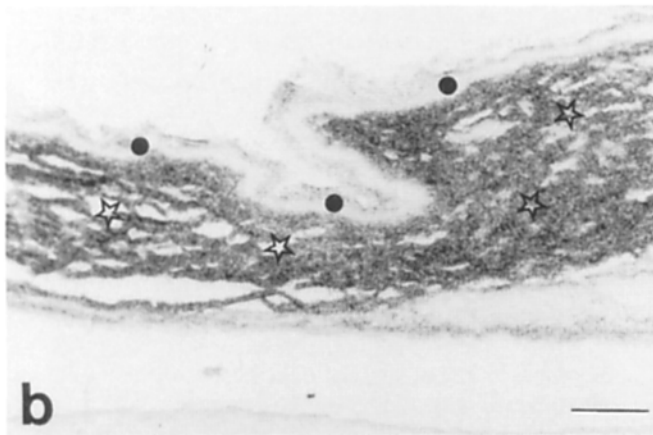
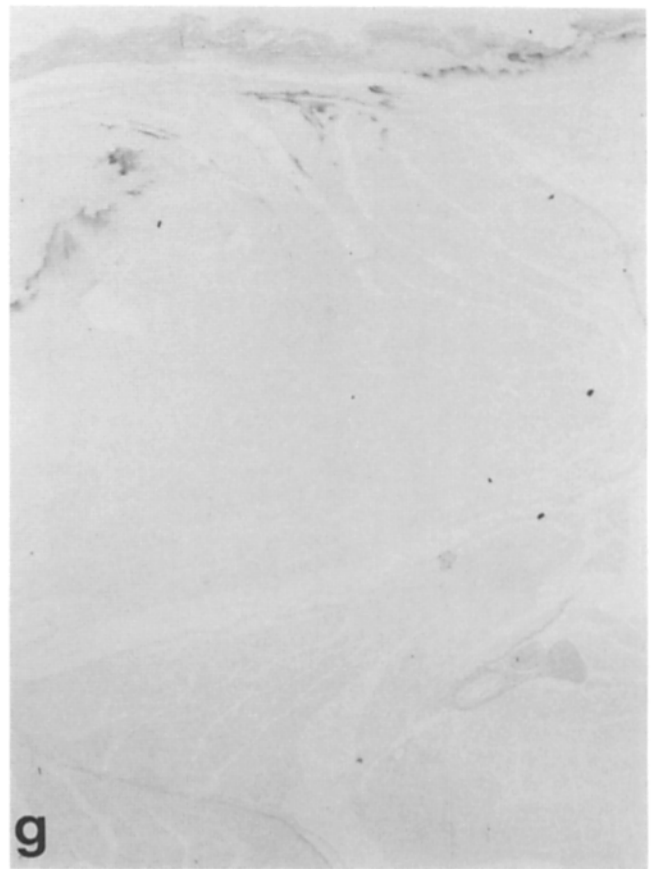
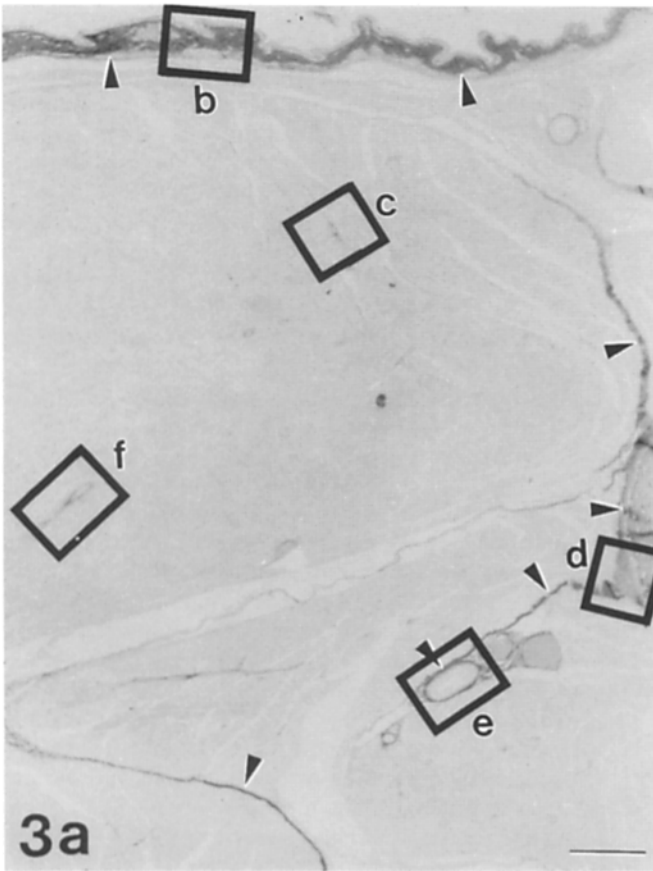
Expression of 9E3 in the Wings of RSV-infected Chicks

With the concentration of virus used in this study, wings injected with RSV develop tumors after ~10 d. Therefore, we labeled wings 11–12 d after injection of the virus or after wounding of infected birds. In wings with primary tumors, 9E3 mRNA was found in the same tissues as in normal wings (Fig. 3) but it was more abundant adjacent to the tumor (Fig. 3, b–e) and decreased with distance from the tumor. However, this message was absent from the tumor tissue itself (Fig. 3 f); only background signal was found except in the connective tissue around blood vessels where expression was comparable to that of vessels in the tissue adjacent to the tumor (compare with Fig. 3 e). The controls, performed on consecutive sections with a probe transcribed from the vector, were negative (Fig. 3, g–i). In wings with wound-induced tumors, 9E3 expression was identical to that in wings with primary tumors, i.e., the tumor itself showed only background signal but the tissues adjacent to the tumor had high levels of 9E3 mRNA (not shown).

Colocalization of 9E3 mRNA and Interstitial Collagen

The pattern of expression of 9E3 in normal tissues coincides with that of collagen in the skin, connective tissue and bone, as judged by Masson's Trichrome staining (Fig. 4, a–c). Furthermore, the elevated expression of 9E3 in the vicinity of wounds (see Fig. 2, b and c) and tumors (see Fig. 3) correlates with the increased deposition of collagen in these areas (Fig. 4, d and e), whereas both 9E3 mRNA and collagen are absent in tumor tissue (Fig. 4, f and g).

To test the possibility that the probe for 9E3 mRNA might



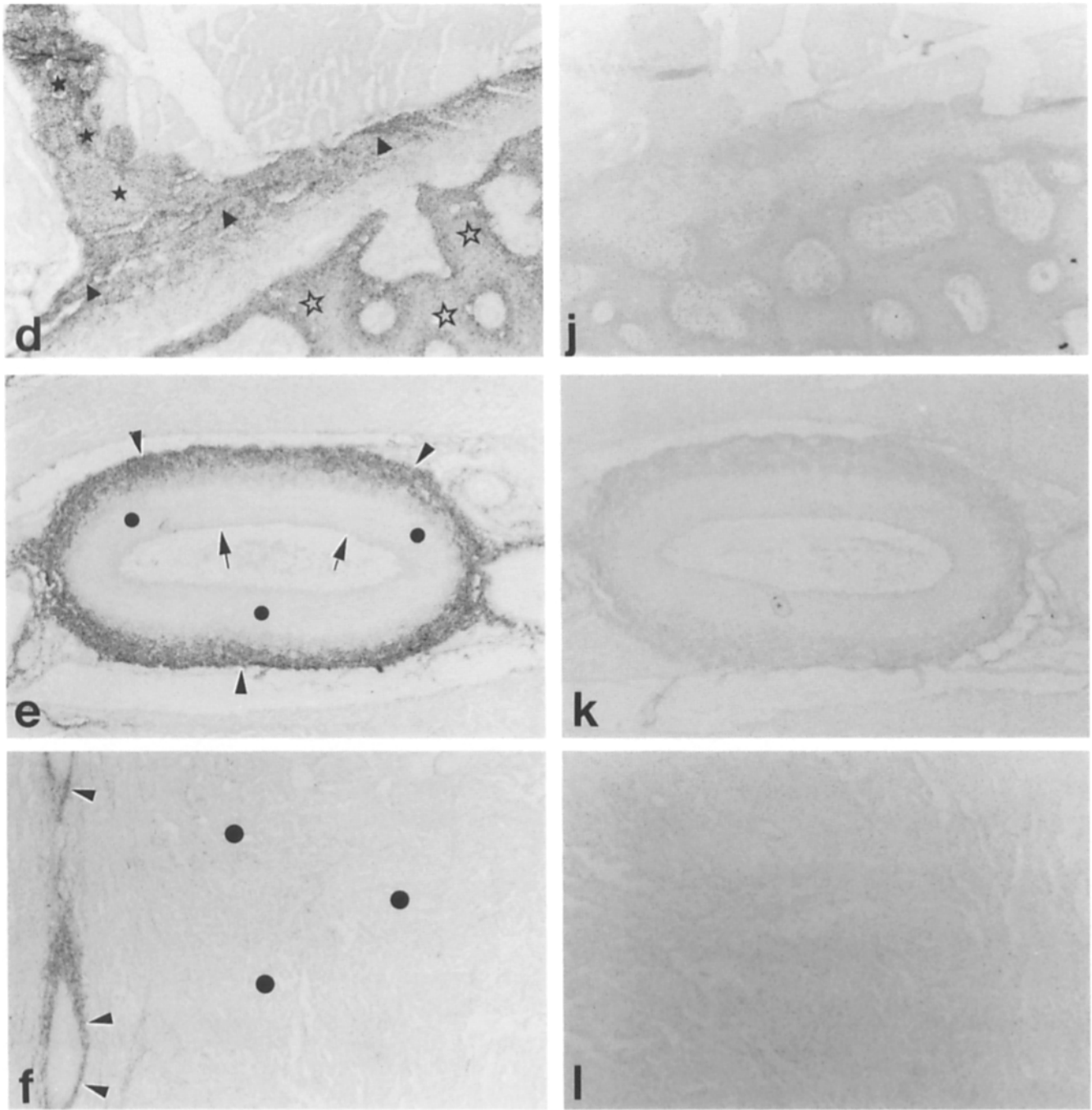
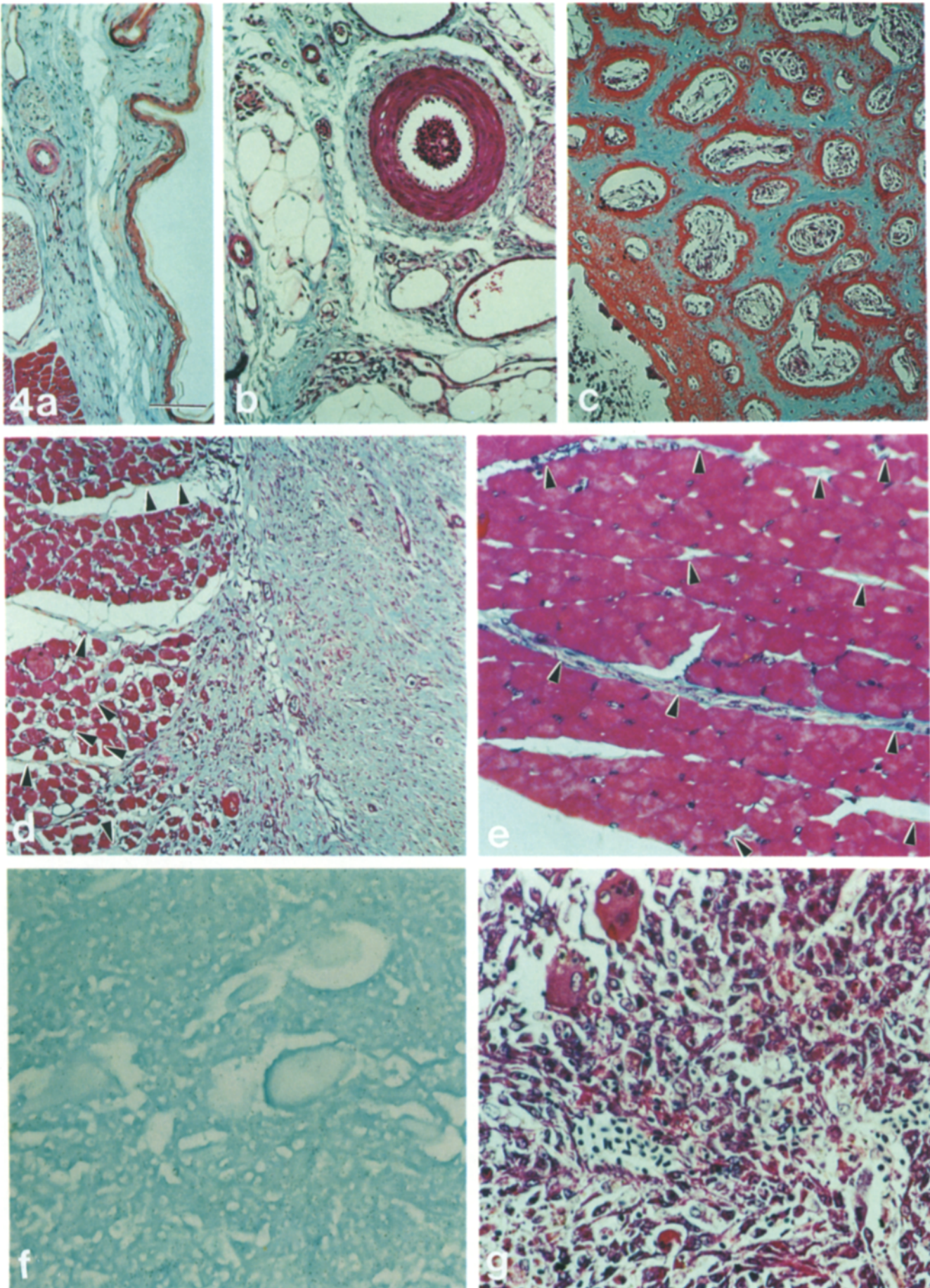


Figure 3. Cross-sections through the wing of a 2-wk-old chick 11 d after being injected with RSV. In situ hybridization was performed using a probe for 9E3 mRNA; *a-f* and for the pGEM1 vector *g-l*. (*a*) Low magnification of wing tissues; dark areas (*arrowheads*) represent the sites of hybridization of the probe with 9E3 mRNA; note that here and in *b-f*, 9E3 mRNA is more abundant than in normal skin (compare with Fig. 1). (*b*) Detail of box "b" in (*a*) showing hybridization in the dermis of the skin (*asterisks*) overlying the tumor tissue and lack of hybridization in the epidermis (*dots*). (*c*) Detail of box "c" in (*a*) showing the presence of 9E3 mRNA in the connective tissue surrounding the muscle blocks (*arrowheads*). (*d*) Detail of box "d" in (*a*) showing abundant 9E3 mRNA in bone (*asterisks*), periosteum (*triangles*), and tendon (*stars*). (*e*) Detail of box "e" in (*a*) showing 9E3 expression in the connective tissue (*arrowheads*) around a blood vessel but absence of expression in smooth muscle (*dots*) and endothelial cells (*arrows*). (*f*) Detail of box "f" in (*a*) showing the absence of 9E3 mRNA in the tumor itself (*dots*) but its presence in the connective tissue of a blood vessel within the tumor (*arrowheads*). (*g*) Section consecutive to that shown in *a* but hybridized with a control probe (pGEM1 vector). Note that the skin, bone, tendon and connective tissue of blood vessels do not appear dark, indicating absence of hybridization. (*h-l*) Enlargements of areas identical to those boxed in *a* for comparison with *b-f* showing in detail that the control is negative. Scale bar, 50 μ m for *a* and *g* and 50 μ m (*b-f*) and (*h-l*).



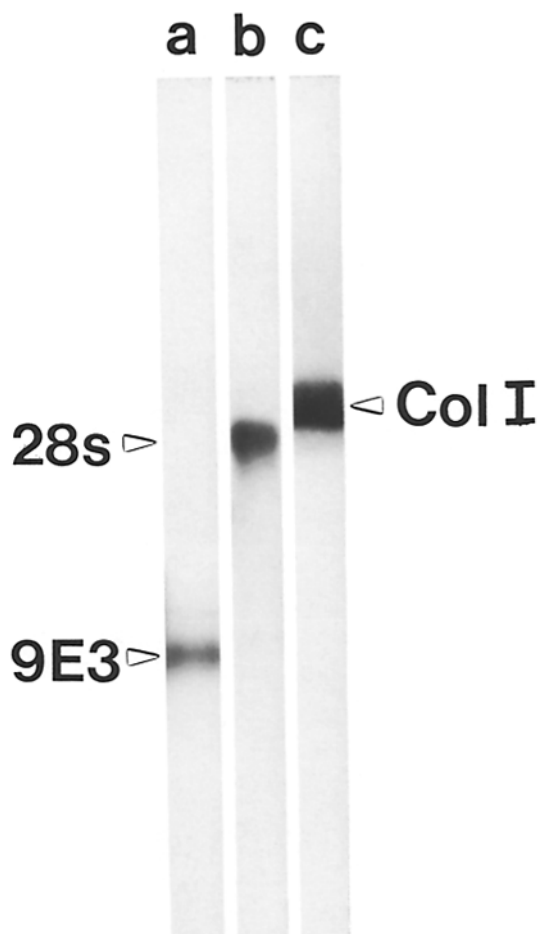


Figure 5. Northern blot containing total RNA isolated from T-CEFs. Lane *a* shows that hybridization with a cDNA probe for 9E3 mRNA labels only the 1.2-kb band characteristic of this mRNA. Lane *b* shows the same RNA hybridized with 28S rRNA for scale. Lane *c* gives the same RNA hybridized with a probe for a portion of the collagen I gene showing hybridization to a 4.0-kb band. Therefore, this probe and that for 9E3 do not cross-react.

crossreact with the collagen message, northern blots containing total RNA isolated from T-CEFs in culture were hybridized with the 9E3 probe. Only a 1.2-kb message was revealed (Fig. 5, lane *a*). The same blot was reprobed for 28S rRNA and for collagen I mRNA (Fig. 5, lanes *b* and *c*). The collagen probe hybridized only to a band of ~4.0 kb, hence the possibility of crossreactivity was ruled out.

We also observed that 9E3 mRNA (Fig. 6, *a* and *d*) and collagen (Fig. 6, *b* and *e*) are more abundant in areas where neovascularization is occurring. To demonstrate this, we la-

beled the tissue with antibodies against von Willebrand factor (von Beust et al., 1988; Yablonka-Reuveni, 1989), which specifically labels endothelial cells. Regions of elevation of expression of 9E3 correlate strongly with areas where new blood vessels are forming (compare Fig. 6, *a* and *d* with Fig. 6, *c* and *f*).

Expression of 9E3 in Cultured Cells

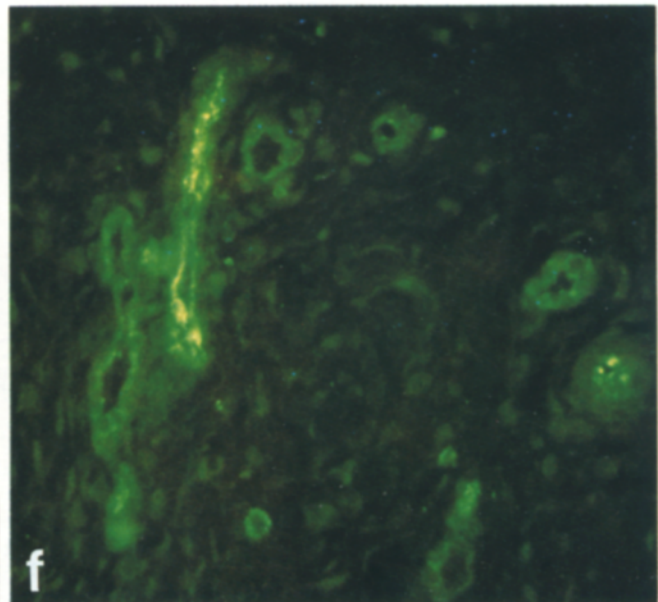
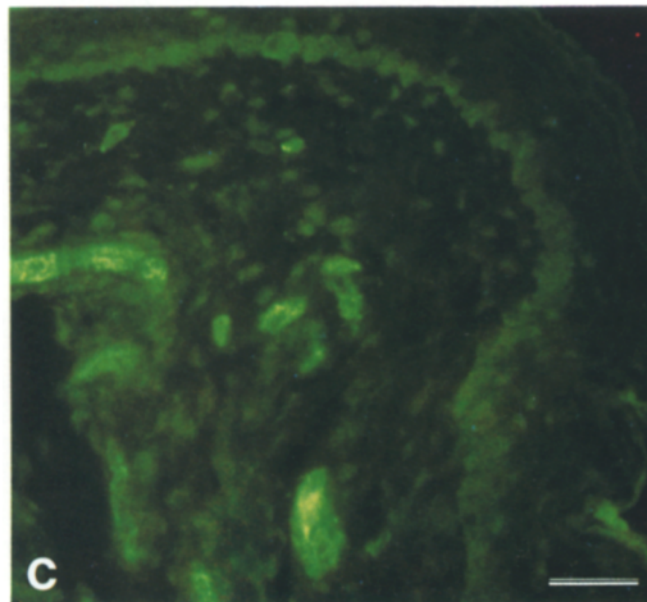
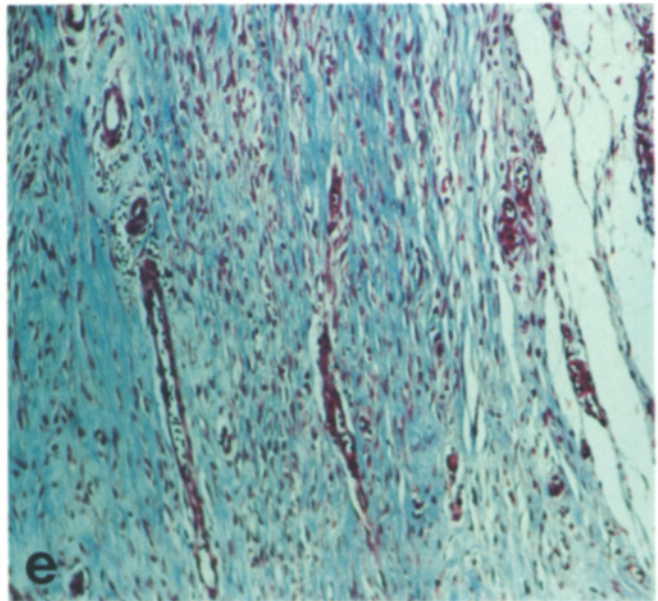
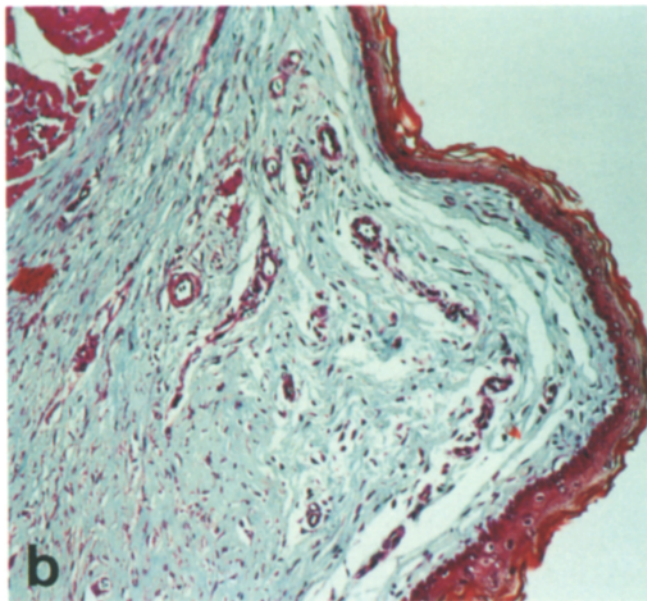
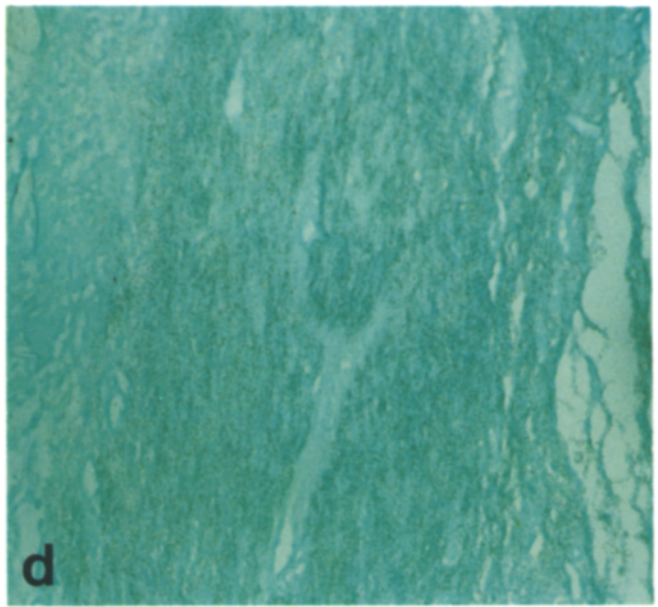
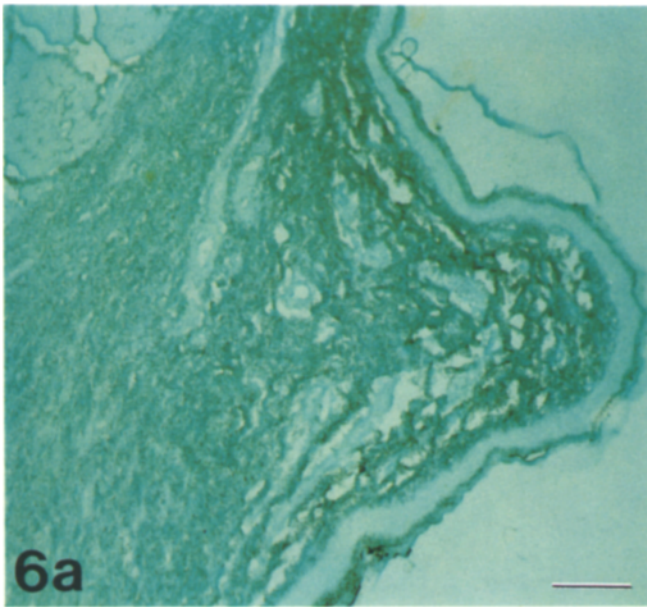
The contrast between the absence of expression of 9E3 in tumor cells in vivo and the very high level of expression in T-CEFs in culture (Sugano et al., 1987; Bedard et al., 1987) led us to investigate the possibility that environmental conditions might influence 9E3 expression. To address this point, it was necessary to investigate whether the differences of expression observed by Sugano et al. (1987) and Bedard et al. (1987) were due to differences in the level of cellular expression under different culture conditions or whether they were due to differences in the fraction of cells that express the gene. To establish a basis for direct comparison between CEFs in culture and tissues in vivo, we performed in situ hybridization on cultured cells using: (*a*) serum-starved confluent CEFs; (*b*) serum-stimulated confluent CEFs; (*c*) CEFs plated at low density; and (*d*) fully transformed CEFs. Serum-starved confluent CEFs showed no detectable levels of 9E3 mRNA (Fig. 7 *a*). However, when these cultures were stimulated by serum factors (5%), about one-third of the cells showed abundant message (Fig. 7 *b*). About half of the CEFs plated at low density expressed 9E3 (Fig. 7 *c*) and the message declined as the cells became confluent, whereas virtually all T-CEFs expressed this gene at very high levels (Fig. 7 *d*). These results are fully consistent with those obtained by Northern blot analysis reported by Sugano et al. (1987) and Bedard et al. (1987). In addition, the data indicate that not all normal cells express 9E3 at the same time.

We also explanted cells from the central region of four advanced tumors and cultured the cells from individual tumors under the same conditions as T-CEFs. Although the cultured tumor cells were fully infected, as indicated by immunostaining with an antibody against the viral protein p19 (Fig. 8 *a*), and transformed, as judged by morphological appearance, 9E3 expression was undetectable (Fig. 8 *b*). We used T-CEFs as positive controls for 9E3 expression (see Fig. 7 *d*).

Time Course Expression of 9E3 in Injected and Wounded Wings

To determine whether or not there may be a transient overexpression of 9E3 associated with the viral infection we followed the expression of the gene by isolating total RNA from wing tissues as a function of time after injection of the virus. Slot blots prepared from RNA extracted from the area of in-

Figure 4. Cross-sections through wings of 2-wk-old chicks. Sections shown in *a–e* and *g* were stained by Masson's Trichrome method (which stains collagen blue, nuclei black, and cytoplasm and cytoskeletal elements red) and the section shown in *f* was labeled for 9E3 by in situ hybridization. (*a*) Normal skin shows the presence of collagen in the dermis and absence in the epidermis. (*b*) Connective tissue between and around blood vessels shows abundant collagen. (*c*) Collagen staining is pronounced in the areas between the lacunae of the bone. (*d*) Collagen is abundant in wound tissue (*right*) and, in the adjacent muscle, it also is abundant in the connective tissue surrounding muscle blocks and individual muscle fibers (*arrowheads*). (*e*) Muscle adjacent to primary tumors also shows enhanced deposition of collagen between the muscle blocks (*arrowheads*). (*f* and *g*) Tumor tissue is negative both for 9E3 expression and for collagen staining (*g*). Note that the pattern of collagen staining coincides with that of 9E3 expression (compare with Figs. 2 and 3). Scale bars, 50 μ m.



jection (Fig. 9 *a*, lane 1) showed little or no elevation of expression at early time points but a rise to higher levels after ~10 days, when a marked increase in the rate of tumor growth was observed. RNA prepared from the center of the tumors without the surrounding tissue was negative (not shown). A similar series collected from wounded wings showed initially high expression which decreased after 24 h but remained somewhat elevated throughout the 16-d experiment (Fig. 9 *a*, lane 2). The expression in tissues associated with wound tumors showed aspects of both the early wound reaction and the later tumor response (not shown). Controls showing the levels of expression in T-CEFs, CEFs, and normal tissue are shown in Fig. 9 *b*.

Discussion

The results presented here show for the first time the expression of the 9E3 gene *in vivo*. In normal tissues, 9E3 mRNA is found at high levels in connective tissue and other tissues of mesenchymal origin such as bone and tendon, and it is overexpressed in response to injury, whether the injury is caused by wounding or by tumor formation. Despite expression at very high levels in T-CEFs in culture, 9E3 is not expressed in RSV-induced tumor cells *in vivo* or in culture.

When considering our results *in vivo*, it must be kept in mind that we are working with newly hatched chicks; a great deal of growth is taking place during the period of our observations (the chicks double in size in 1 wk). In normal wings, 9E3 expression is found mostly in those tissues that are actively growing by cell division and also actively synthesizing extracellular matrix components. That is, the expression of this gene is high in the connective tissue and other tissues of mesenchymal origin, with the exception of muscle and endothelium. It is possible, therefore, that the protein for this gene (9 kD) is a growth factor involved in regulation of normal growth, as suggested for the *gro* gene by Anisowicz et al. (1987, 1988). The marked enhancement of expression of 9E3 at the wound site and in adjacent tissues suggests that the product of this gene may play a role in wound response and/or repair.

It is intriguing to note that 9E3 expression occurs in the same tissues that stain for transforming growth factor- β_1 (TGF- β_1) in the mouse embryo (Heine et al., 1987), pointing to possible similarities in function or a causal relationship. The codistribution of 9E3-specific mRNA with collagen in connective tissue and bone suggests that the protein for this gene could potentially play a role in promoting and/or stabilizing matrix deposition or that matrix could be important for 9E3 expression. The elevation of expression of 9E3 in wounded areas and the enhanced deposition of collagen in the same regions provide further support for this notion. The codistribution of TGF- β and 9E3 expression becomes more interesting in view of these observations because it is known that TGF- β induces a marked increase in collagen deposition and accelerates wound healing (Mus-

toe et al., 1987). On the other hand, TGF- β has been shown to prevent the EGF-induced expression of KC in endothelial cells (Takehara et al., 1987). Although 9E3 is not expressed in endothelial cells, this observation points to the extreme complexity governing the expression of these various genes. Further work is needed to establish whether or not there is a relationship between TGF- β and the product of the 9E3 gene. It also is possible that the protein may act as an angiogenic factor because 9E3 expression remains elevated in areas of neovascularization.

The lack of expression of 9E3 in tumor cells both *in vivo* and in culture indicates that activation of this gene is not required for maintenance of the transformed phenotype. Furthermore, the time course experiments using RNA extracted from the tissues at the site of virus injection (Fig. 9 *a*, lane 1), showed no apparent increase in gene expression at early time points, suggesting that viral infection *per se* does not stimulate expression of 9E3. However, RNA blots are not sensitive enough to rule out definitively the possibility of expression in a small subpopulation of cells. Therefore, before any involvement of 9E3 in induction of transformation is ruled out, it may be necessary to perform *in situ* hybridization at very early times after virus injection to test this possibility.

The elevation of 9E3 around the periphery of tumors could simply reflect a response to injury analogous to the elevation around wounds; increase in expression of 9E3 could be a direct response to growth factors secreted by the tumor cells. Activation of the 9E3 gene in the normal cells surrounding the tumor might, in turn, facilitate tumor growth. Although we do not have direct evidence for this scenario, it is consistent with the results presented by Vogel et al. (1988). These investigators studied the expression of the *tat* gene of the human immunodeficiency virus (HIV) in transgenic mice. They found expression of the *tat* gene in Northern blots prepared from skin containing dermal lesions that resemble Kaposi's sarcoma. However, when the tumor cells were explanted and grown in culture they could not detect *tat* mRNA. The authors concluded that *in situ* hybridization studies would be necessary to establish whether the *tat* mRNA detected in the northern blots was indeed due to expression of the gene in tumor tissue or whether it was due to expression in other cells surrounding the skin lesions (as we find for 9E3). Since Kaposi's sarcoma develops only in mice that are transgenic for the *tat* gene, the product of this gene must in some way be involved in tumor formation. It is possible, therefore, that 9E3 could act in a similar way on RSV-induced tumors.

In the work presented here, we have found a disparity between the overexpression of 9E3 in T-CEFs in culture and the absence of expression in tumor cells both *in vivo* and in culture. This lack of correspondence shows that there are intrinsic differences between T-CEFs in culture and the RSV target cell populations *in vivo*. Despite the early promise of cell culture as a well-defined and simple system to study growth

Figure 6. Cross-sections through the wing of a 2-wk-old chick. Sections shown in *a* and *d* were labeled by *in situ* hybridization of 9E3; those shown in *b* and *e* were stained for collagen by Masson's Trichrome method; those shown in *c* and *f* were immunostained with anti-von Willebrand factor antibody to label endothelial cells. Overexpression of 9E3 in skin (*a*) and granulation tissue (*d*) coincides with abundant deposition of collagen (*b* and *e*) in those areas and with increased neovascularization (*c* and *f*) in the same tissues. Scale bars, 50 μ m.

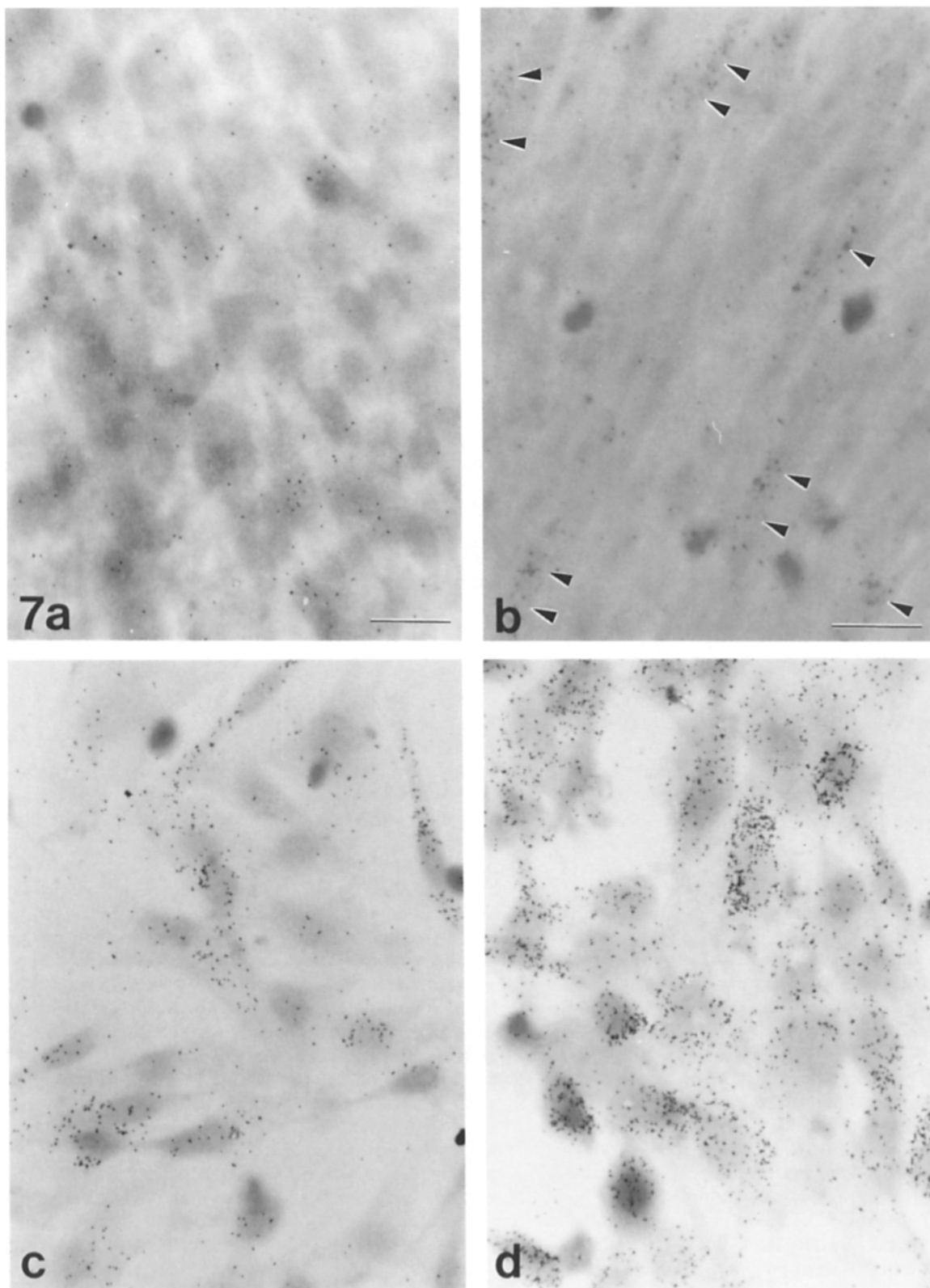


Figure 7. In situ hybridization for 9E3 mRNA in CEFs in culture. (a) Confluent CEFs under starving conditions show essentially no expression. (b) Confluent CEFs kept under starving conditions and then stimulated by change of medium containing 5% serum show a number of cells expressing 9E3 mRNA (arrowheads). (c) CEFs plated at low density show about half of the cells expressing 9E3 mRNA. (d) Transformed CEFs show nearly all cells expressing abundant 9E3 mRNA. Scale bars, 50 μ m.

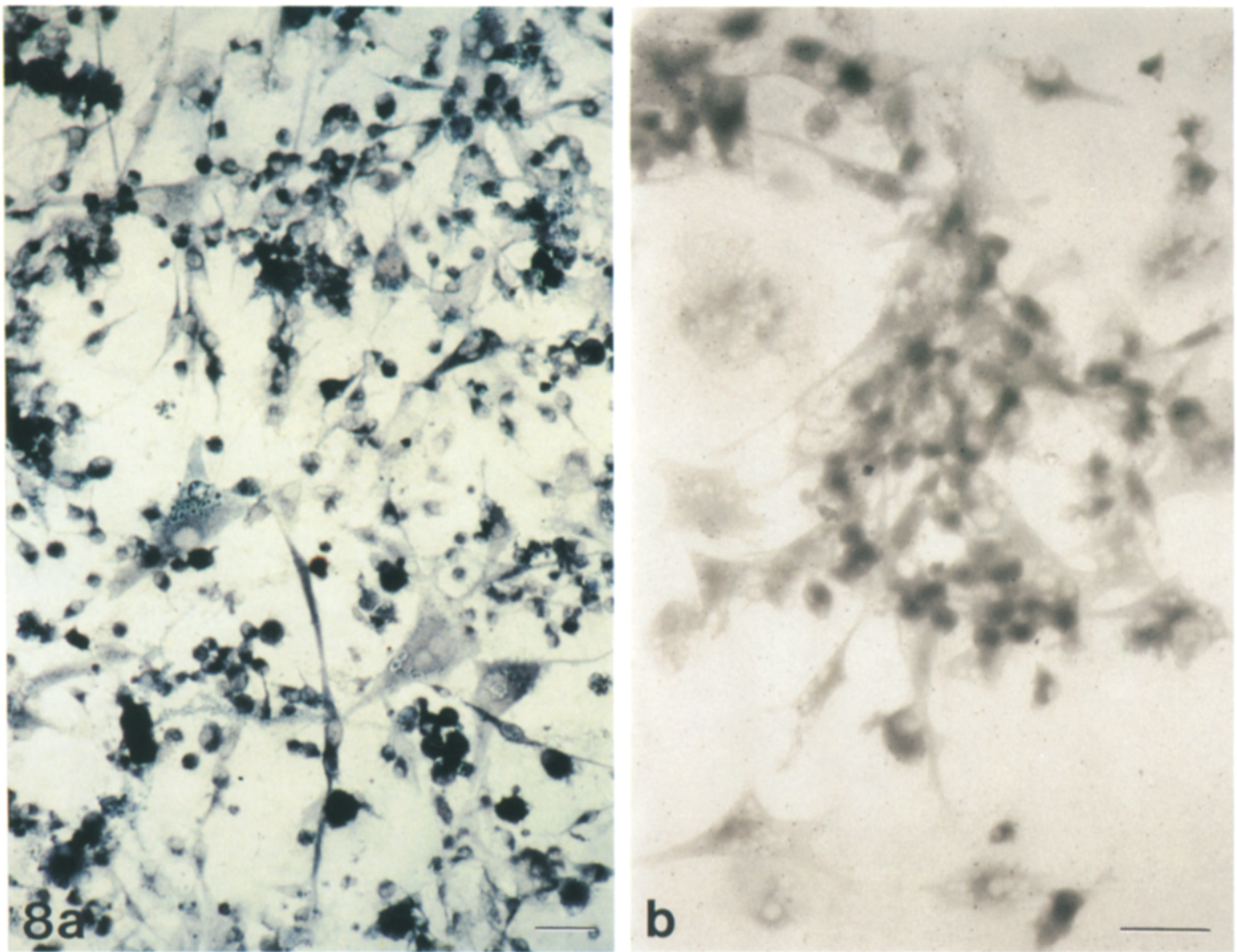


Figure 8. Cells taken from advanced tumors and cultured on plastic under the same conditions as T-CEFs of Fig. 7 *d*. (*a*) Immunolabeling with an antibody against p19^{ras} and alkaline phosphatase using the avidin-biotin method (Howlett et al., 1987; Stoker and Bissell, 1987). Most cells show the presence of the protein indicating that they are infected with the virus. They also show the characteristic morphology of transformed cells. (*b*) In situ hybridization for 9E3 mRNA. As was the case in vivo (Fig. 3 *f*), the culture tumor cells do not express 9E3. Scale bars, 50 μ m.

factors and viral oncogenes, it is becoming increasingly clear that a combined in vivo-in vitro approach is necessary to unravel the biological significance of factors whose existence and identities have been established in culture (for review, see Bissell, 1981). To understand RSV tumorigenicity, therefore, it is important that the relevance of transformation studies in culture be ascertained by studies of tumor formation in vivo.

In conclusion, our results indicate that 9E3 plays a physiological role in normal tissues, perhaps as a growth regulator, and that elevation of expression of the gene upon injury may be important in wound response and/or repair. Furthermore, the absence of expression of this gene in tumor cells in vivo or in culture suggests that this gene does not play a role in tumor maintenance but the elevated expression at the edges of the tumor could implicate a role in tumor growth.

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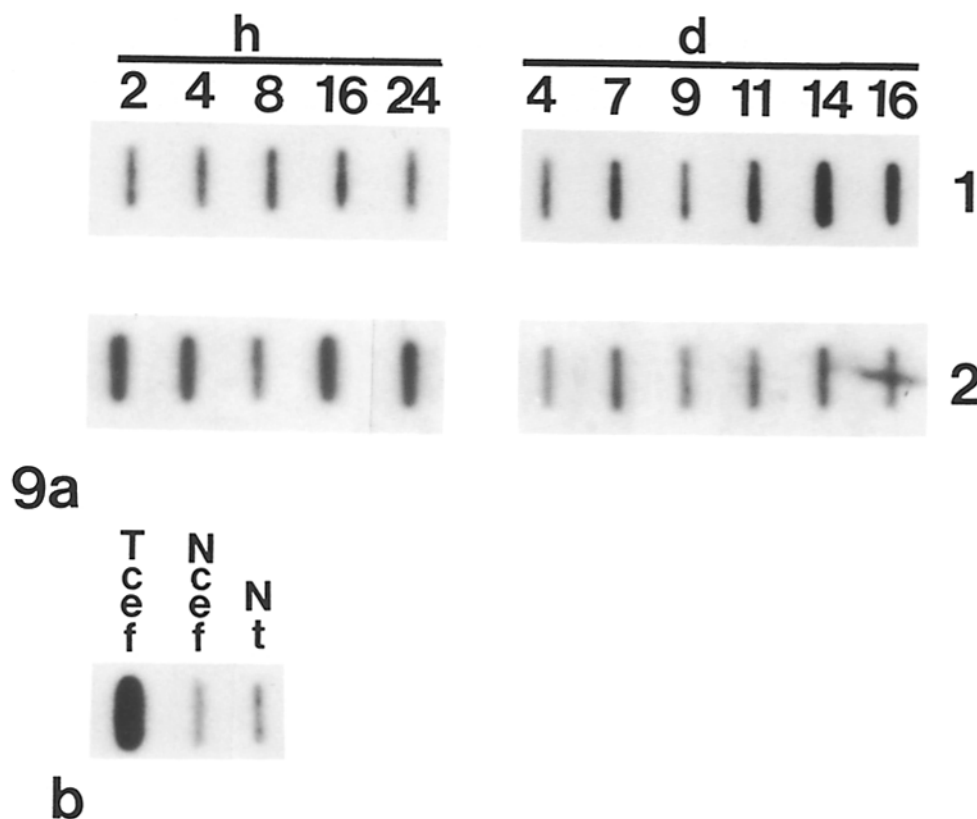


Figure 9. Slot blots containing total RNA extracted from wing tissues and cells in culture. (a) Tissues collected at successive times after injection of the virus (lane 1) or insertion of a clip (lane 2). Lane 1, the tissues collected before a palpable tumor appears (up to 9 d after injection of the virus) show no appreciable elevated levels of 9E3 expression. Once tumors appear, they grow rapidly and by day 16 a large mass has formed. The tumor cells themselves do not express 9E3 (Figs. 3f and 8), hence the elevation of expression after day 11 represents the overexpression of 9E3 in tissues adjacent to the tumor. Lane 2, the tissues collected during the first 24 h after wounding show marked elevation in 9E3 expression. Expression declines thereafter. (b) Total RNA extracted from transformed CEFs (*Tcef*), normal CEFs (*Ncef*), and normal wing tissue (*Nt*).

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